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EFEITOS DA ALOPREGNANOLONA SOBRE O COMPORTAMENTO TIPO DEPRESSIVO DE RATOS COM PERFIS DISTINTOS DE IMOBILIDADE NO NADO FORÇADO

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RESUMO

A depressão é um transtorno mental altamente prevalente e incapacitante cuja etiologia está relacionada a fatores ambientais e individuais. Apesar de tratável, a eficácia das terapias farmacológicas antidepressivas ainda é incompleta. Muitos estudos recentes têm tentado esclarecer a fisiopatologia da depressão a fim de prover terapias farmacológicas mais eficazes. Alguns destes estudos focam na elucidação dos mecanismos genéticos que concedem predisposição ou resiliência à depressão e outros têm procurado expandir o escopo das investigações relacionadas aos sistemas neuroquímicos que estão envolvidos na depressão. Os neuroesteroides, como a alopregnanolona, têm recebido atenção devido à sua ação como moduladores do sistema GABAérgico, sendo intimamente relacionados com a depressão em estudos clínicos e pré-clínicos. O objetivo deste estudo foi verificar o efeito da alopregnanolona em ratos com diferentes padrões de comportamentos tipo-depressivos gerados através de cruzamentos seletivos. Neste trabalho, ratos Wistar machos e fêmeas foram submetidos ao teste do nado forçado (FST) por três gerações para selecionar os animais com perfis extremos de imobilidade e cruzá-los entre si, dando origem a duas subpopulações com históricos "familiares" distintos de comportamentos tipo-depressivos: alta ou baixa imobilidade. Machos de cada subgrupo da terceira geração receberam infusão intracerebroventricular de alopregnanolona, veículo (controle negativo) ou imipramina (controle positivo) e avaliados novamente no FST. Os subgrupos dos machos na terceira geração tiveram um tempo de imobilidade estatisticamente diferente entre si. No entanto, apenas os ratos de alta imobilidade diferiram da população inicial. Tanto a imipramina quanto a alopregnanolona mostraram um efeito tipo-antidepressivo nos animais de alta imobilidade, ainda que em magnitudes diferentes. Estas drogas não foram capazes de alterar o tempo de imobilidade no FST nos animais de baixa imobilidade. Estes resultados apontam para um efeito diferenciado dos antidepressivos dependente da predisposição a apresentar comportamentos tipo-depressivos.

Palavras-chave: depressão maior; hereditariedade; receptor $GABA_A$

ABSTRACT

Depression is a highly prevalent and incapacitating disorder with an etiology that is related to environmental and individual factors. Though treatable, efficacy of currently available pharmacological therapies is still incomplete. Several studies have tried to clarify the pathophysiology of depression aiming to provide more efficient pharmacological therapies. Some of these studies focus on the elucidation of the genetic mechanisms that underlie predisposition or resilience to depression, and others have tried to expand the scope of investigations related to the neurochemical systems involved in depression. Neurosteroids, such as allopregnanolone, have received special attention due to their action as modulators of the GABAergic system, being linked to depression in clinical and pre-clinical studies. This study aimed to verify the effect of allopregnanolone in rats with different patterns of depressive-like behaviors obtained through selective breeding. Male and female Wistar rats were submitted to the forced swim test (FST) for three generations to select animals with extreme immobility profiles and inbreed them, giving origin to two subpopulations with different backgrounds of depressive-like behaviors: high or low immobility. Male rats of both subgroups in the third generation received intracerebroventricular infusion of either allopregnanolone, vehicle (negative control) or imipramine (positive control) and tested again in the FST. Immobility groups in the third generation had different immobility durations. However, only high immobility rats differed from the initial population. Both imipramine and allopregnanolone showed an antidepressive-like effect in high immobility animals, though in different magnitudes. These drugs were not able to change immobility duration in the FST on low immobility rats. These results point to a different antidepressant effect depending on the predisposition to present depressive-like behaviors

Keywords: major depression; heredity; $GABA_A$ receptor

LISTA DE ABREVIATURAS

GWAS – *Genome-wide association studies*; Estudo de associação ampla do genoma

GABA – Ácido γ-aminobutírico

SNC – Sistema nervoso central

MAO – Monoamino oxidase

ISRS – Inibidor seletivo da recaptação de serotonina

$GABA_A$-R – Receptor $GABA_A$

TSPO – *Translocator protein 18 kDa*; Proteína translocadora 18 kDa

FST – *Forced swim test*; teste do nado forçado

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1. INTRODUÇÃO

1.1 Depressão

A depressão é um transtorno altamente prevalente, acometendo cerca de 322 milhões de pessoas no mundo, o que corresponde a aproximadamente 4,4% da população mundial (FERRARI et al., 2013). Também é altamente incapacitante, com níveis de incapacidade superiores a condições médicas crônicas como diabetes, hipertensão ou artrite (RAKEL, 1999), sendo atualmente considerada a maior causa de incapacidade no mundo (WHO, 2017). Este transtorno mental caracteriza-se, de forma geral, por profundo e duradouro sentimento de tristeza, desolação e desesperança. Apesar de seus sintomas mais característicos serem de cunho psíquico – como humor depressivo, perda da capacidade de sentir prazer em atividades que antes eram agradáveis, fadiga e diminuição da capacidade de concentração –, também são frequentes sintomas fisiológicos – como alterações do sono, do apetite, do interesse sexual, e do ciclo circadiano (DEL PORTO, 1999). Seu diagnóstico clínico é definido pelo Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders*; DSM) que, em sua quinta e mais atual edição, requer que um paciente apresente 5 de 9 sintomas (humor depressivo, anedonia, mudanças no apetite ou peso; alterações no sono, retardo ou agitação psicomotora, fadiga, sentimento de culpa ou desvalorização e dificuldade de concentração) durante ao menos 2 semanas ininterruptas, com prejuízo a seu rendimento normal (BENTLEY; PAGALILAUAN; SIMPSON, 2014).

A etiologia da depressão, embora venha sendo estudada há muitos anos, ainda não foi completamente elucidada. O que se sabe até o momento é que ela é causada por um conjunto complexo de fatores, que podem ser primariamente divididos em externos (ambientais) e internos (individuais). Acontecimentos como morte de um parente, perda de emprego ou status social, separação de cônjuge ou crises financeiras atuam de maneira a gerar uma resposta adaptativa e transitória de humor deprimido. No entanto, mesmo na ausência de fatores externos, fatores internos podem resultar em uma resposta maladaptativa, dando origem aos sintomas característicos da depressão maior (ver Figura 1) (BERGSTROM; MEACHAM, 2016).

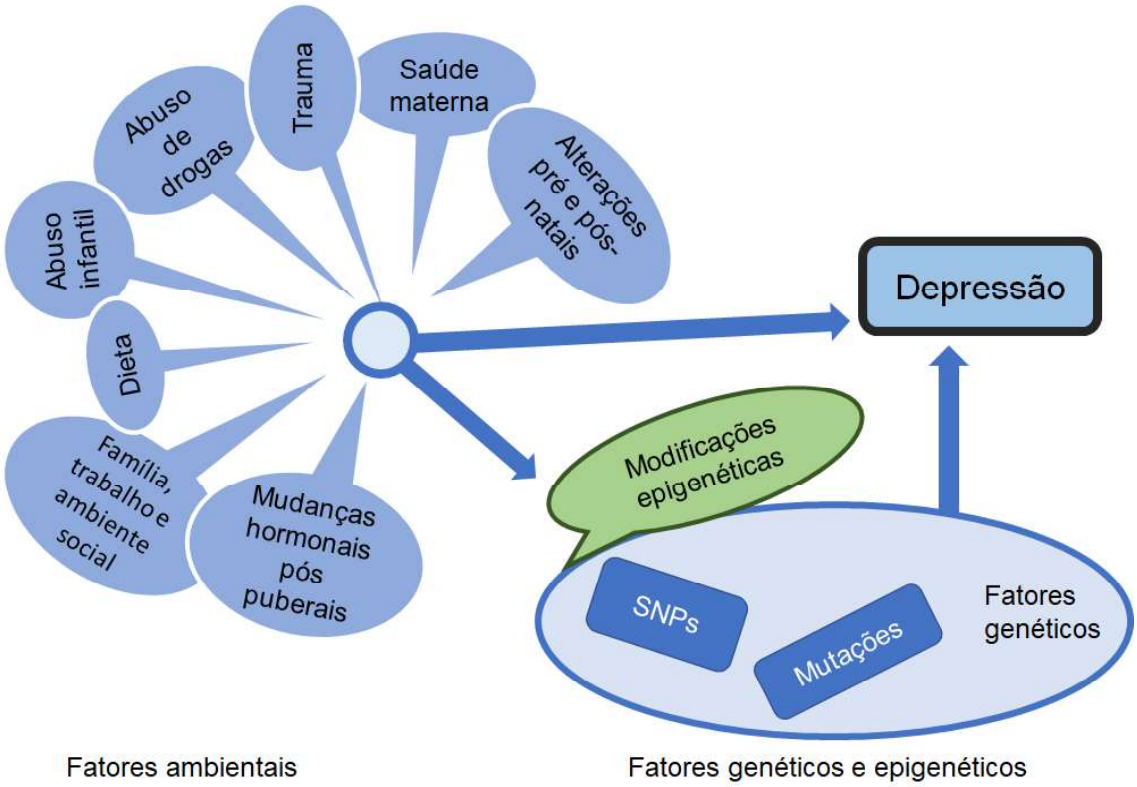

Figura 1: Representação esquemática da etiologia multifatorial da depressão. Fatores ambientais como trauma, dieta, pressões sociais, abuso de drogas, etc. interagem com fatores genéticos de um indivíduo e, caso estes fatores contribuam para uma predisposição, podem gerar mudanças epigenéticas que levam ao desenvolvimento do transtorno depressivo. Figura adaptada de Roy e colaboradores (2011).

1.1.1 Aspectos genéticos da depressão

Dentro do escopo dos chamados fatores internos ou individuais da depressão, o mais claro destes é, provavelmente, o genético. A genética da depressão pode ser primariamente observada através de sua heritabilidade, que é de aproximadamente 37% (SULLIVAN; NEALE; KENDLER, 2000). Este transtorno, portanto, possui importante ligação com histórico familiar, já que parentes próximos de indivíduos diagnosticados com depressão maior apresentam níveis de depressão e ansiedade elevados (WATTERS et al., 2013) e um risco maior de desenvolver ansiedade e depressão (WILDE et al., 2014). Estes achados levaram ao questionamento de quais seriam os genes implicados na maior susceptibilidade à depressão. Ao longo do tempo, polimorfismos em diversos genes têm sido sugeridos como possíveis marcadores para o desenvolvimento de depressão com base em suas funções

conhecidas, como algumas proteínas neurotróficas (em especial o BDNF e seus receptores) e receptores de alguns neurotransmissores, como o receptor 5-HT de serotonina. No entanto, até o momento nenhum teve seu papel confirmado (ROY et al., 2014). Mais recentemente, estudos de associação ampla do genoma (*genome-wide association studies*; GWAS) têm sido utilizados para realizar o raciocínio inverso, levantando possíveis polimorfismos relacionados à depressão através do sequenciamento completo do genoma de milhares de casos e controles. Estes estudos, infelizmente, também foram incapazes de obter resultados conclusivos até o momento (DUNN et al., 2015). Por outro lado, a análise combinada de polimorfismos levantados neste tipo de estudo aumenta a chance de acerto na previsão de desenvolvimento de transtornos psiquiátricos, incluindo a depressão (MAIER et al., 2015).

1.1.2 Modelos animais genéticos de depressão

Alguns trabalhos têm buscado simular este conceito na pesquisa com animais ao criar modelos genéticos através do cruzamento sucessivo de animais que apresentem determinada característica comportamental análoga a comportamentos depressivos. No início dos anos 1990, foram publicados trabalhos estudando ratos congenitamente suscetíveis ou resilientes ao protocolo do desamparo aprendido, o que foi realizado através do cruzamento seletivo de animais com o perfil desejado por mais de 23 gerações (HENN; EDWARDS, 1991; LACHMAN et al., 1992). No final da mesma década, a vocalização ultrassônica infantil por separação maternal foi utilizada como comportamento de interesse para o cruzamento seletivo por 5 gerações (BRUNELLI et al., 1997). Além disso, Weiss e colaboradores cruzaram ratos Sprague-Dawley com perfis extremos de imobilidade em uma versão modificada do teste do nado forçado (somente uma sessão de 15 minutos) por 18 gerações. Este estudo encontrou uma resposta antidepressiva acentuada a um tratamento crônico com desipramina nos ratos mais imóveis (WEISS; CIERPIAL; WEST, 1998). Anos mais tarde, foram produzidas linhagens de ratos Wistar-Kyoto selecionados no teste do nado forçado e cruzados durante mais de 7 gerações. O tratamento subagudo nestes animais com desipramina e fenelzina, mas não fluoxetina, provocou uma significativa redução no comportamento tipo-depressivo dos ratos de alta imobilidade. Somente a

fenelzina provocou este efeito nos ratos de baixa imobilidade, mas numa magnitude muito menor (WILL; AIRD; REDEI, 2003). El Yacoubi e colaboradores desenvolveram, em 2003, um modelo em camundongos através do cruzamento sucessivo de animais por 14 gerações com pontuações extremas de imobilidade no teste de suspensão de cauda. O tratamento agudo com antidepressivos tricíclicos e inibidores da recaptação de serotonina provocaram um efeito tipo-antidepressivo nos animais de alta imobilidade e o tratamento crônico com fluoxetina também reduziu a imobilidade nestes ratos, mas não nos de baixa imobilidade (EL YACOUBI et al., 2003). Um trabalho mais recente que cruzou ratos Sprague-Dawley por 10 gerações utilizou um critério multifatorial para a seleção comportamental, baseando-se não apenas na imobilidade no teste do nado forçado, mas também na preferência por açúcar e locomoção na gaiola. Interessantemente, não foi observado nenhum efeito de antidepressivos no comportamento ou expressão de BDNF na linhagem de animais mais tipo-deprimidos. Apenas terapia eletroconvulsiva produziu estes efeitos (GERSNER et al., 2014).

Tabela 1: Modelos animais de depressão gerados através do cruzamento seletivo comportamental

Publicado em	Espécie	Teste(s) comportamental(is)	Número de gerações	Denominação
HENN; EDWARDS, 1991	ratos (Sprague-Dawley)	desamparo aprendido	23	cLH e cNLH
BRUNELLI et al., 1997	ratos (N:NIH)	vocalização ultrassônica por separação maternal	5	Linhagem alta e linhagem baixa
WEISS; CIERPIAL; WEST, 1998	ratos (Sprague-Dawley)	teste do nado forçado	18	SwHi e SwLo
WILL; AIRD; REDEI, 2003	ratos (Wistar-Kyoto)	teste do nado forçado	7	WMI e WLI
EL YACOUBI et al., 2003	camundongos (CD1)	teste de suspensão de cauda	14	H/Rouen e NH/Rouen
GERSNER et al., 2014	ratos (Sprague-Dawley)	teste do nado forçado; preferência por sacarose; ambulação total	10	DRL e MRL

Um aspecto que chama atenção nestes estudos proponentes de modelos animais genéticos de depressão, compilados na Tabela 1, é o número elevado de gerações nas quais os animais são sucessivamente cruzados com base na seleção comportamental tipo-depressiva. Estes números parecem contrastar com os estudos em humanos que investigam a hereditariedade da depressão ao demonstrar com êxito o risco elevado de sintomas e distúrbios depressivos em parentes de primeiro grau de indivíduos com depressão (SULLIVAN et al., 2000; WATTERS et al., 2013; WILDE et al., 2014). Além disso, diferenças comportamentais claras tendem a aparecer logo nas primeiras gerações dos estudos com animais (EL YACOUBI et al., 2003; WEISS et al., 1998; WILL et al., 2003), indicando que um número tão alto de gerações possa ser, em última análise, desnecessário para a aplicação do modelo em testes farmacológicos. Além disso, os tratamentos farmacológicos testados nestes modelos até o momento se restringem aos fármacos de clássica aplicação clínica (especialmente antidepressivos tricíclicos e inibidores seletivos da recaptação da serotonina), gerando uma oportunidade de testar novos alvos farmacológicos e compreender melhor os sistemas neuroquímicos envolvidos na depressão.

1.1.3 Aspectos neuroquímicos da depressão

Outro aspecto essencial no estudo da fisiopatologia da depressão é a sua neuroquímica, ou seja, quais alterações estão presentes no sistema nervoso central (SNC) de indivíduos acometidos com este transtorno. Esta questão é particularmente importante pois de seu estudo provém o desenvolvimento de terapias farmacológicas efetivas contra a doença. Por meio de estudos investigando os efeitos neuroquímicos de fármacos com efeito agonista ou antagonista de diferentes sistemas de neurotransmissores, foi possível esclarecer os papeis dos mesmos na fisiopatologia da depressão. Através da verificação indireta da participação de um sistema neurotransmissor na neuroquímica da depressão, neurotransmissores como a serotonina, noradrenalina, dopamina (HAMON; BLIER, 2013) e o ácido γ-aminobutírico (GABA) (MOHLER, 2012) acabaram por ganhar destaque dentro deste contexto. De maneira geral, pode-se dizer que o conhecimento atualmente disponível envolvendo a fisiopatologia da depressão está fundamentado nos

efeitos neuroquímicos provocados pelos agentes terapêuticos utilizados em seu tratamento (KALIA, 2005).

Durante muitos anos, as investigações acerca da etiologia da depressão ficaram restritas às teorias monoaminérgicas. Estas, por sua vez, se baseiam no efeito das monoaminas – grupo de neurotransmissores formado pela dopamina, noradrenalina (estas, catecolaminas) e serotonina – sobre os neurônios SNC através de seus receptores específicos (MULINARI, 2012). A origem deste conceito surge em decorrência da primeira terapia farmacológica aplicada com sucesso no tratamento da depressão, utilizando-se de inibidores da enzima monoamino oxidase (MAO) para aliviar os sintomas depressivos no início da década de 1950. Ao observar que a elevação das catecolaminas através do uso de inibidores da MAO ou outras drogas como a imipramina, uma hipótese catecolaminérgica da neuroquímica da depressão ganhou força nos anos 60. A imipramina, um antidepressivo tricíclico, também classificado como antidepressivo clássico, ocasiona bloqueio da recaptação de catecolaminas (noradrenalina e dopamina), serotonina, assim como é antagonista de receptores H1, M e alfa2. Com o passar do tempo, a presença de efeitos colaterais importantes deste fármaco e de outros deste grupo levou ao desenvolvimento de novas classes de antidepressivos mais seletivas.

Mais precisamente, o papel da serotonina na sintomatologia da depressão tornou-se evidente, migrando o foco de uma hipótese catecolaminérgica para uma hipótese serotoninérgica. Isto se consolidou com o advento dos inibidores seletivos da recaptação de serotonina (ISRSs) no tratamento da depressão, sendo atualmente os fármacos de escolha e mais comumente prescritos para o tratamento da doença (revisado em LOPEZ-MUNOZ; ALAMO, 2009). No entanto, apesar de décadas de pesquisa e desenvolvimento de novos fármacos, estudos de meta-análise demonstraram que a eficácia dos antidepressivos, independentemente do mecanismo de ação, ainda é incompleta, sendo que aproximadamente um terço dos pacientes não responde à medicação (KIRSCH et al., 2008; TURNER et al., 2008). Este cenário sugere que é necessário ampliar os sistemas de neurotransmissores implicados na depressão e investigar sua participação na fisiopatologia da mesma. O paradigma monoaminérgico parece ser responsável por apenas parte da neuroquímica deste distúrbio, abrindo caminho para a implicação de

outros sistemas de neurotransmissores na depressão (BERTON; NESTLER, 2006).

O sistema GABAérgico e seu envolvimento nas desordens do humor começaram a ser discutidos há 25 anos quando o ácido valproico – um agonista GABAérgico – apresentou resultados clínicos positivos no tratamento do transtorno de bipolaridade (EMRICH et al., 1980). Ensaios clínicos subsequentes demonstraram que pacientes com depressão possuíam níveis plasmáticos de GABA diminuídos (PETTY et al., 1992), sendo que estes níveis estão diretamente correlacionados com as concentrações de GABA tanto na matriz extracelular do SNC quanto no líquido cefalorraquidiano (PETTY et al., 1993). Outros estudos clínicos foram capazes de demonstrar também que o grau de severidade da depressão possui uma correlação inversa com os níveis de GABA no líquido cefalorraquidiano (GERNER; HARE, 1981) e no plasma (KUCUKIBRAHIMOGLU et al., 2009). Estes achados foram corroborados em estudos utilizando animais, demonstrando que os comportamentos tipo-depressivos no teste do nado forçado e os níveis extracelulares de GABA estão inversamente correlacionados em ratos (GOMEZ; BARROS, 2003) e que o tratamento com antidepressivos aumenta a concentração estriatal de GABA em ratos (PARENT et al., 2002). Por outro lado, ainda não está claro qual é o agente causal nesta relação entre as concentrações de GABA medidas nestas condições e as manifestações comportamentais observadas nos animais (MOHLER, 2012).

Dentre os diversos agonistas do sistema GABAérgico, os mais conhecidos são provavelmente os benzodiazepínicos e o etanol – além do GABA, naturalmente. Nos últimos tempos, no entanto, o grupo dos neuroesteroides tem recebido atenção crescente, sendo objeto de diversos estudos para determinar como exatamente ocorre a sua modulação do sistema GABAérgico. Os achados recentes relacionados aos neuroesteroides tem sido promissores, levantando diversas hipóteses relacionadas à possibilidade do desenvolvimento de novas terapias farmacológicas para o tratamento de distúrbios do humor baseadas nestas vias neuroquímicas (BELELLI; LAMBERT, 2005; CARTA; BHAT; PRETI, 2012; RUPPRECHT; HOLSBOER, 1999).

1.2 Neuroesteroides

Neuroesteroides são substâncias sintetizadas no sistema nervoso – em especial no SNC – a partir de hormônios esteroides circulantes ou do colesterol (CARTA et al., 2012). Eles fazem parte de uma classe mais ampla conhecida como “esteroides neuroativos”, que também inclui agentes endógenos similares produzidos fora do sistema nervoso central e substâncias sintéticas que têm ações no sistema nervoso central similar às dos neuroesteroides (PAUL; PURDY, 1992). Sua síntese ocorre tanto em células gliais (especialmente astrócitos) quanto em neurônios (especialmente células de Purkinje) do sistema nervoso central e periférico (PAUL; PURDY, 1992), assim como na glândula hipófise (TSUTSUI; HARAGUCHI, 2014). Os neuroesteroides são importantes agonistas do sistema GABAérgico, tendo o receptor GABA tipo A ($GABA_A$-R) como um de seus principais alvos (ROBEL; BAULIEU, 1994).

1.2.1 Modulação do receptor $GABA_A$

O $GABA_A$-R é o principal mediador de inibição no SNC, sendo parte fundamental no mecanismo de redução da excitabilidade neuronal. O $GABA_A$-R consiste em um canal iônico transmembrana que, quando ativado pelo GABA ou outro ligante que exerça função semelhante, permite a entrada de íons de Cl^- nos neurônios. Este influxo leva estes neurônios a um estado de hiperpolarização no qual sua excitabilidade encontra-se reduzida e, consequentemente, o disparo de potenciais de ação dos mesmos é dificultado (CARVER; REDDY, 2013). O $GABA_A$-R é uma proteína pentamérica, contendo cinco subunidades transmembrana que se distribuem em torno do canal iônico que confere função ao receptor (como pode ser observado na Figura 2), sendo que o seu arranjo influencia na sua função e em sua afinidade com ligantes (WANG, 2011).

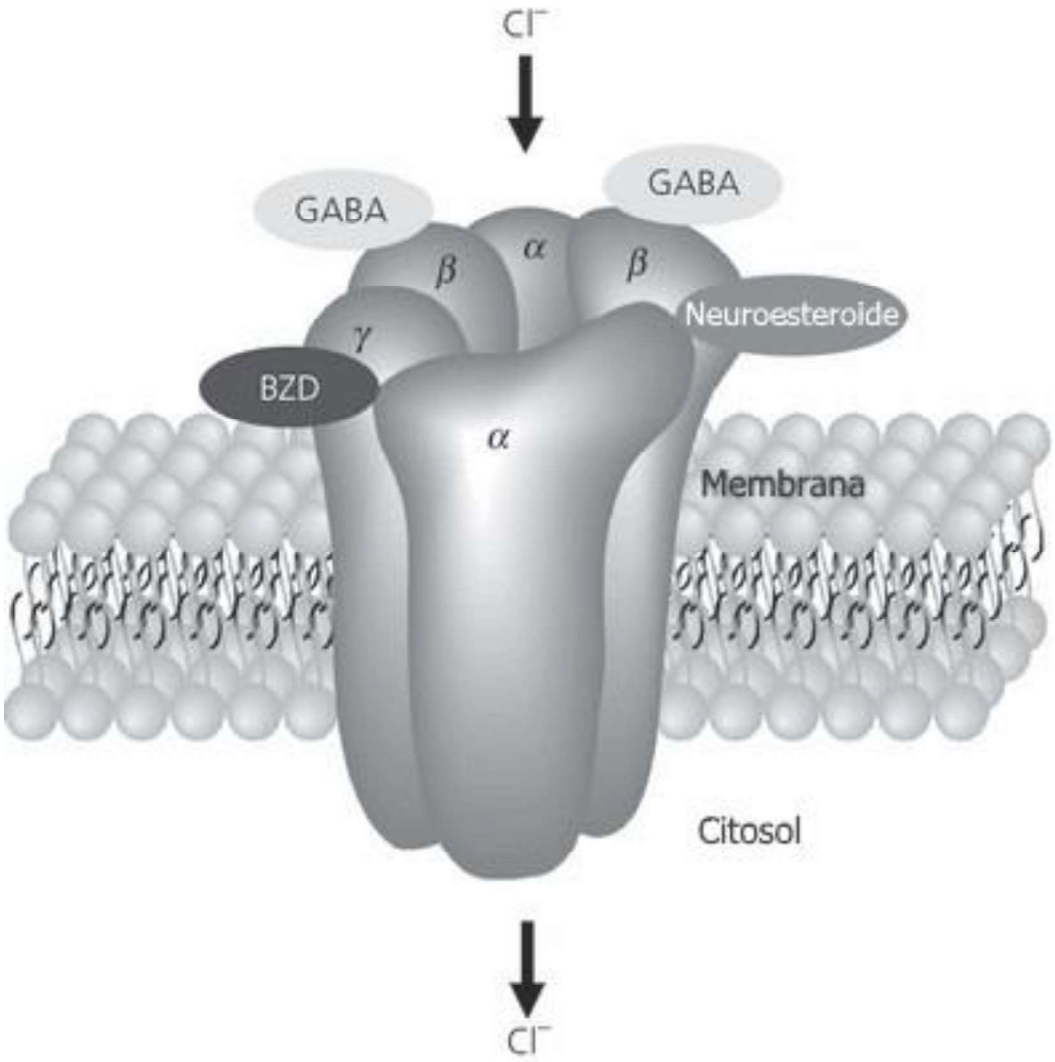

Figura 2: Representação do receptor GABAA com destaque para os sítios de ligação do GABA, benzodiazepínicos (BZD) e neuroesteroides. Figura adaptada de Nothdurfter e colaboradores (2011).

Até o momento, 19 diferentes subunidades do $GABA_A$-R já foram descritas, sendo elas: α1–6, β1–3, δ, ε, γ1–4, π, θ e ρ1-3 (HEVERS; LUDDENS, 1998; OLSEN; SIEGHART, 2009). Sendo o $GABA_A$-R uma proteína pentamérica, não é possível um único $GABA_A$-R contenha todas as subunidades conhecidas até o momento. Assim, existe um imenso potencial de combinações que podem ser formadas, cada uma com diferentes graus de afinidade por diferentes ligantes (CARVER; REDDY, 2013; FRITSCHY; MOHLER, 1995; JECHLINGER et al., 1998; SIEGHART et al., 1999; WISDEN et al., 1992). No entanto, é importante notar que as subunidades mais prevalentes nos $GABA_A$-Rs de ratos são as α, β, γ e δ. Além disto, estes

receptores em geral apresentam ao menos duas subunidades α, duas subunidades β e uma subunidade γ ou δ, resultando em uma proporção de aproximadamente 60% dos $GABA_A$-Rs expressando α1β2γ2, 15 a 20% expressando α2βγ2, 5% α4βδ ou α4βγ e menos de 5% expressando α5β2γ2 ou α6β2/3γ2 (RUDOLPH; KNOFLACH, 2011). Esta diferenciação é relevante pois sua combinação tem efeito sobre sua função e localização. Os receptores que contém a subunidade δ, por exemplo, atuam na inibição tônica por serem extrassinápticos, ao contrário dos receptores contendo a subunidade γ, que atuam na inibição fásica na fenda sináptica (WANG, 2011). E é justamente sobre estes receptores extrassinápticos contendo a subunidade δ que os neuroesteroides parecem exercer a maior parte de sua função (STELL et al., 2003).

1.2.2 Relação com a depressão

Clinicamente, a relação dos neuroesteroides com a depressão tem sido evidenciada com bastante sucesso. O uso de fármacos como a finasterida, que diminuem a produção de neuroesteroides, parece estar associado com o aparecimento de sintomas depressivos quando aplicados para o tratamento de alopecia ou hiperplasia prostática em humanos (ALTOMARE; CAPELLA, 2002; IRWIG, 2012; RAHIMI-ARDABILI et al., 2006). Devido à prevalência superior de depressão em mulheres, as flutuações de progesterona - um esteroide neuroativo e precursor da alopregnanolona - durante o ciclo menstrual e a gravidez também foram objeto de investigação para esclarecer se os neuroesteroides contribuem para depressão perimenstrual e pós-parto. No ciclo menstrual, a progesterona circula em níveis baixos durante a fase folicular, mas se eleva consideravelmente durante a fase lútea. Na gravidez, os níveis de progesterona aumentam rapidamente e atingem seu pico no terceiro trimestre, reduzindo para níveis basais um dia após o parto. Os baixos níveis observados durante estes momentos específicos, fase folicular e fase puerperal, se correlacionam com um risco elevado para aparecimento de humor depressivo em mulheres (FRYE et al., 2011). No mesmo sentido, um estudo piloto demonstrou que mulheres com transtorno disfórico pré-menstrual possuem sensibilidade alterada à administração exógena de alopregnanolona (TIMBY et al., 2016), um dos metabólitos da progesterona que têm sido amplamente

estudado e se mostrado um importante candidato ao neuroesteroide com o principal papel na modulação da depressão: a alopregnanolona (ZORUMSKI et al., 2013).

1.3 Alopregnanolona

A alopregnanolona (outras denominações conhecidas: 3α-hidroxi-5α-pregnan-20-ona; 3α,5α-tetrahidroprogesterona; 3α,5α-TH PROG; 3α,5α-THP) é um neuroesteroide que está entre as substâncias endógenas com maior potencial modulador alostérico positivo do $GABA_A$-R (BAULIEU; ROBEL; SCHUMACHER, 2001; MAJEWSKA et al., 1986; PUIA et al., 1990; RUPPRECHT, 2003; VAN BROEKHOVEN; VERKES, 2003). Os mecanismos desta modulação são dependentes da concentração de alopregnanolona presentes no espaço extracelular. Quando presente em concentrações nanomolares, a alopregnanolona potencializa o de cloro nos canais iônicos induzidos pelo GABA (MAJEWSKA et al., 1986; PAUL; PURDY, 1992), prolongando a duração do seu estímulo inibitório (AKK et al., 2010; HARRISON et al., 1987; LAMBERT et al., 1995). Se presente em concentrações micromolares, a alopregnanolona é capaz de induzir diretamente a abertura dos canais de Cl^- constituídos nos receptores $GABA_A$ (PAUL; PURDY, 1992).

No mesmo sentido, a alopregnanolona potencializa os estímulos inibitórios mediados não apenas pelo GABA sobre o $GABA_A$-R, mas também por outros ligantes ativadores diretos e moduladores alostéricos positivos deste receptor, como por exemplo os benzodiazepínicos (GUIDOTTI et al., 2001; MATSUMOTO et al., 1999; PINNA et al., 2000; PUIA et al., 2003). A alopregnanolona possui um sítio de ligação ao $GABA_A$-R formado pelas subunidades α e β (HOSIE et al., 2006), mas recentemente foi proposto que seu sítio de ligação mais importante está localizado na subunidade α, através do qual a alopregnanolona é capaz de provocar a ativação do receptor $GABA_A$ sem haver necessariamente uma ligação conjunta com o GABA (BRACAMONTES et al., 2011). Por outro lado, já foi demonstrado que a potenciação causada pela alopregnanolona não depende de uma subunidade específica, já que seu sítio de ligação encontrado na subunidade α pode ser movido para subunidades β2 ou γ2.(BRACAMONTES et al., 2012).

1.3.1 Síntese

A síntese da alopregnanolona, como em outros esteroides, se inicia na metabolização do colesterol. No entanto, os passos que ocorrem com maior importância em áreas cerebrais com relevância para a regulação do humor são aqueles que envolvem o metabolismo da progesterona (DONG et al., 2001) e da 5α-diidroprogesterona (DONG et al., 2001; PINNA et al., 2000). Estas transformações envolvem a ação de duas enzimas: a 5α-redutase, que reduz a progesterona em 5α-diidroprogesterona; e a 3α-hidroxiesteróide oxiredutase, que converte reversivelmente a 5α-diidroprogesterona a alopregnanolona (RUPPRECHT; HOLSBOER, 1999), conforme pode ser observado na Figura 3. Diferentes regiões cerebrais possuem diferentes conteúdos de 5α-diidroprogesterona e alopregnanolona, já que suas distribuições não são uniformes (DONG et al., 2001; PINNA et al., 2000). Em roedores, as maiores concentrações destes neuroesteroides encontram-se no bulbo olfatório, seguido do córtex frontal, hipocampo, amígdala, estriado e cerebelo (PIBIRI et al., 2008). Fatores estressores como o isolamento social em camundongos, no entanto, podem reduzir estas concentrações para até metade dos níveis normais (PIBIRI et al., 2008). Outro fator importante para a síntese de alopregnanolona é a atividade da proteína translocadora 18 kDa (TSPO), cuja função é internalizar o colesterol nas mitocôndrias. Esta proteína é formada por cinco subunidades transmembrana e está localizada na membrana externa de mitocôndrias de células esteroidogênicas. A TSPO tem sido demonstrada como um importante modulador na neuroesteroidogênese e seu papel em distúrbios do humor tem ganhado importância (NOTHDURFTER et al., 2012; PAPADOPOULOS; FAN; ZIRKIN, 2017).

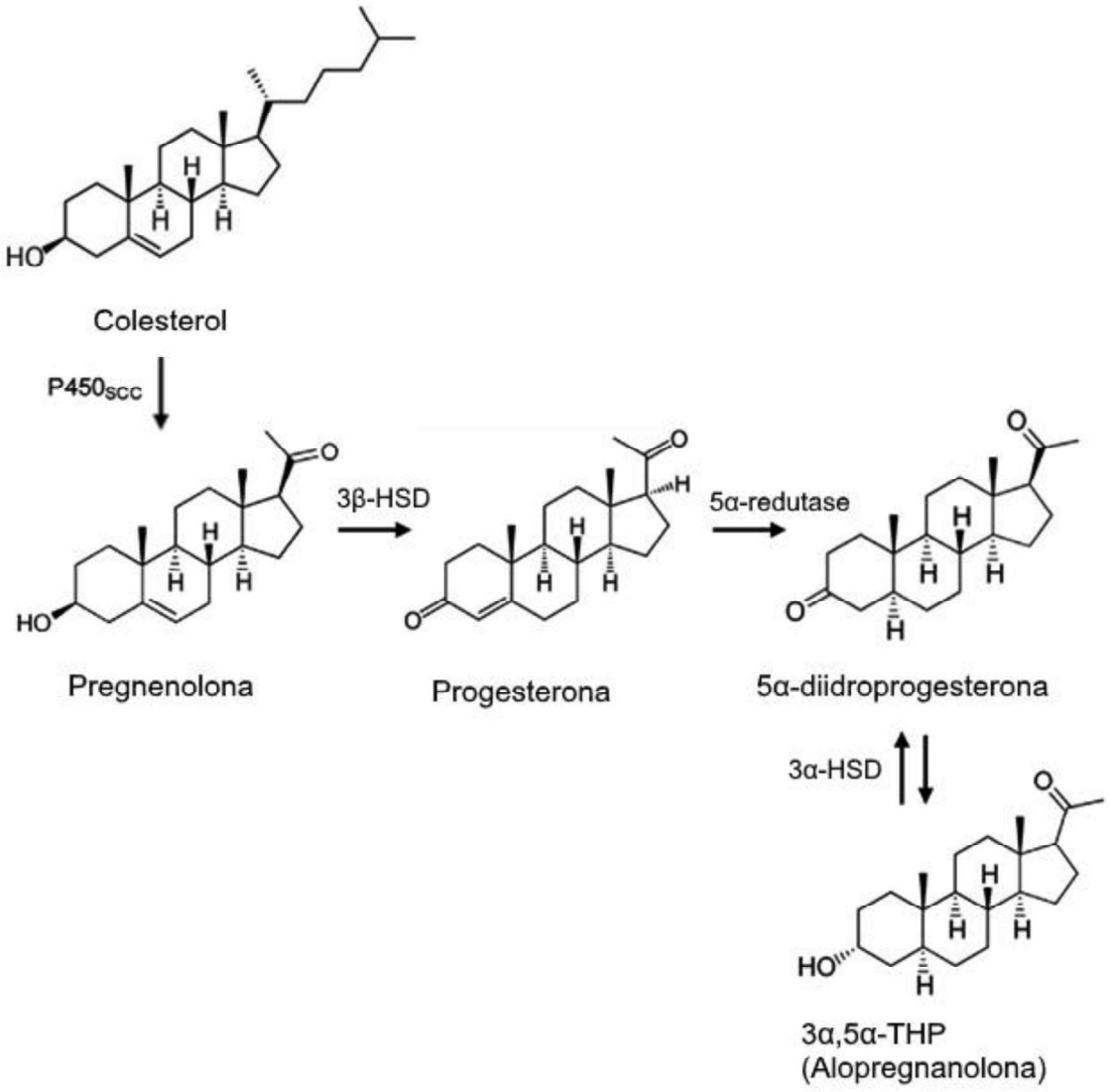

Figura 3: Representação esquemática da síntese da alopregnanonola a partir do colesterol. A pregnenolona é convertida através da enzima 3β-hidroxiesteroide desidrogenase (3β-HSD) em progesterona, que subsequentemente sofre ação da 5α-redutase e passa para a forma de 5α-diidroprogesterona. Esta, por sua vez, passa reversivelmente a alopregnanolona por ação da enzima oxirredutora 3α-Hidroxiesteroide oxidoredutase (3α-HSD). Figura adaptada de Wirth (2011).

1.3.2 Efeitos antidepressivos

A alopregnanolona tem sido amplamente implicada como um importante agente na fisiopatologia de diferentes transtornos do humor relacionados ao estresse como ansiedade, pânico e depressão. Estudos em humanos e em animais demonstraram que seus níveis estão reduzidos em indivíduos acometidos com estes distúrbios (BALI; JAGGI, 2014; SCHULE; NOTHDURFTER; RUPPRECHT, 2014). O estresse e a depressão possuem

uma neurobiologia bastante interligada (GOLD; MACHADO-VIEIRA; PAVLATOU, 2015), sendo que este conceito tem sido amplamente aplicado em animais para a modelagem da depressão através do modelo de estresse crônico moderado, que conta com alta validação comportamental e neuroquímica (HILL et al., 2012). Neste sentido, a alopregnanolona também é apontada como um importante fator de resistência ao estresse, já que a inibição de sua síntese gera comportamentos ansiogênicos em camundongos submetidos a um estímulo estressor agudo (YOSHIZAWA et al., 2017).

O tratamento bem-sucedido com antidepressivos está associado ao aumento dos níveis de alopregnanolona em ratos adrenalectomizados/castrados (UZUNOV et al., 1996). Em humanos, foi observado que os níveis de alopregnanonola de pacientes com depressão unipolar encontram-se reduzidos em comparação com controles não deprimidos e que a administração de fluoxetina ou fluvoxamina é capaz de restaurar os níveis normais deste neuroesteroide (UZUNOVA et al., 1998). Alguns estudos recentes trazem resultados interessantes envolvendo a TSPO e sua relação com a alopregnanolona. A superexpressão da TSPO no giro denteado do hipocampo provoca efeitos tipo-antidepressivos e tipo-ansiolíticos acompanhados de um aumento das concentrações de alopregnanolona na mesma região cerebral de camundongos (LI et al., 2017). O fármaco YL-IPA08, ligante da TSPO, produziu efeitos tipo-antidepressivos e tipo-ansiolíticos em ratos e camundongos (ZHANG et al., 2014) e aumentou as concentrações de alopregnanolona no hipocampo e córtex pré-frontal de ratos submetidos a estresse crônico (ZHANG et al., 2017). Resultados semelhantes foram encontrados com a administração de outro ligante da TSPO (AC-5216), que produziu efeitos tipo-antidepressivos em um modelo animal de depressão induzido por diabetes mellitus tipo 2, em conjunto com um aumento nas concentrações de alopregnanolona no hipocampo e no córtex pré-frontal (QIU et al., 2016).

Algumas áreas cerebrais parecem estar mais importantemente envolvidas no efeito antidepressivo da alopregnanolona. Estudos em animais demonstram alterações no conteúdo de alopregnanolona de acordo com a presença de comportamentos tipo-depressivos e/ou seu tratamento com fármacos de aplicação clínica que regiões cerebrais como o estriado, o córtex

frontal (UZUNOV et al., 1996), o córtex occipital (UZUNOVA et al., 2004), o *nucleus accumbens* (MOLINA-HERNANDEZ et al., 2005; SANCHEZ et al., 2008), e principalmente o hipocampo (FRYE; WALF, 2002; UZUNOV et al., 1996). A infusão intra-hipocampal de alopregnanolona em ratos é capaz de diminuir o tempo de imobilidade no teste do nado forçado e aumentar a expressão de mRNA da subunidade γ2 do $GABA_A$-R na mesma região (NIN et al., 2008). O mesmo ocorre na infusão no *nucleus accumbens*, sendo possível detectar um aumento da expressão gênica das subunidades δ e γ2 do receptor $GABA_A$ no hipocampo direito (NIN et al., 2012). Quando infundida no córtex pré-frontal, a alopregnanolona não parece ser capaz de reduzir o comportamento tipo-depressivo de ratos (NIN, 2012), apesar de aumentar a expressão das subunidades δ e γ2 nesta mesma região (ALMEIDA, 2014). Estes resultados apontam que a alopregnanolona é capaz de exercer seu efeito antidepressivo em múltiplas regiões cerebrais através de sua interação com o $GABA_A$-R.

O efeito antidepressivo da alopregnanolona também foi testado em alguns modelos animais de depressão. Em ratos submetidos ao protocolo de desamparo aprendido, a infusão de alopregnanolona no hipocampo e na amígdala produziu efeitos tipo-antidepressivos (SHIRAYAMA et al., 2011). Os níveis de alopregnanolona também se mostraram reduzidos em um modelo de estresse crônico por isolamento social, sendo que a administração exógena de alopregnanolona produziu um efeito tipo-antidepressivo nestes ratos (EVANS et al., 2012). Em ratos seletivamente cruzados para dar origem a linhagens com alta ou baixa vocalização infantil, foi observado que os níveis hipocampais e amigdalares de alopregnanolona encontravam-se reduzidos na linhagem de alta vocalização em comparação com a linhagem de baixa vocalização (ZIMMERBERG et al., 2005). Não há, no entanto, nenhum estudo até o momento que tenha estudado o efeito da administração exógena de alopregnanolona em um modelo genético de depressão.

2. JUSTIFICATIVA

A depressão é um transtorno altamente prevalente e incapacitante. Apesar de existirem terapias farmacológicas disponíveis para o tratamento da depressão, sua eficácia ainda é incompleta. Isto provavelmente é devido ao fato de que as terapias atualmente disponíveis ainda se baseiam em hipóteses contidas na teoria monoaminérgica da depressão, apesar de já se saber que existem diversos outros sistemas de neurotransmissores envolvidos na patofisilogia deste transtorno além das monoaminas. Os neuroesteroides e sua relação com o sistema GABAérgico formam um abastado campo de estudo para o desenvolvimento de novos agentes farmacológicos para o tratamento da depressão.

Além disso, é importante lembrar que parte da etiologia da depressão é genética, concedendo diferentes graus de susceptibilidade ao desenvolvimento deste distúrbio a pessoas com diferentes históricos familiares. É relevante estudar como este fator influencia o tratamento, especialmente no contexto da busca por novos alvos farmacológicos. Neste sentido, estudos em animais mostram-se úteis nesta pesquisa exploratória e a modelagem dos aspectos hereditários da depressão permite uma investigação mais completa do efeito dos agentes farmacológicos propostos para o tratamento deste distúrbio.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar o efeito da administração intracerebroventricular de alopregnanolona sobre a imobilidade de ratos no teste da natação forçada (FST) com maior e menor predisposição de apresentar comportamentos tipo-depressivos.

3.2 Objetivos específicos

Produzir linhagens de ratos predispostos a apresentar maiores e menores tempos de imobilidade no FST, através de seleção artificial e endocruzamentos dentro destes grupos de animais, por três gerações.

Avaliar o efeito da alopregnanolona sobre o comportamento de animais de maiores e menores tempos de imobilidade no FST.

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The effect of intracerebroventricular allopregnanolone on depressive-like behaviors of rats selectively bred for high and low immobility in the forced swim test

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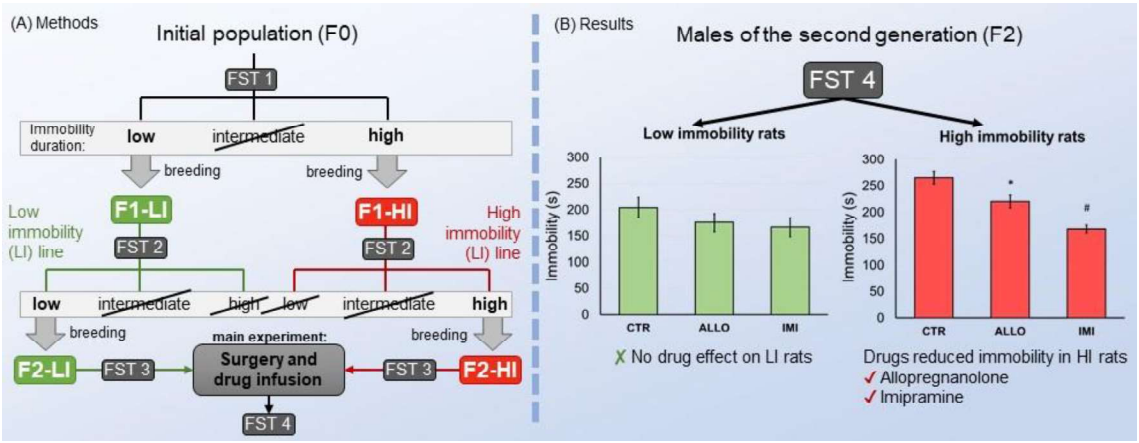
Highlights:

- Rats with high (HI) or low immobility (LI) in the forced swim were selectively bred
- These two strains differed behaviorally from each other after three generations
- Allopregnanolone and imipramine reduced depressive-like behavior in HI rats

Graphical abstract:



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Abstract

Depression is a highly incapacitating disorder known to have a multifactorial etiology, including a hereditary genetic background. The neurosteroid allopregnanolone (ALLO) interacts with the $GABA_A$ receptors as a positive modulator and has been shown to have an antidepressant-like effect in animals. This study aimed to assess ALLO effects in animals with different backgrounds of depressive-like activity. An initial population (F0) of male and female Wistar rats was submitted to the Forced Swim Test (FST) for screening of the immobility behavior. Rats with extreme immobility times were selected for the High Immobility (HI) group and Low Immobility (LI) group for breeding, giving origin to the subsequent generations F1 and F2. F2 males were submitted to stereotaxic surgery for lateral ventricle cannulation and intracerebroventricular infusion of 5 µg/rat of ALLO, 5 µg/rat of imipramine (IMI) or vehicle (CTR). The intracerebroventricular infusions occurred 24, 5 and 1 hour prior to the test sessions of the FST. We observed that 3rd generation rats from the HI and LI groups had significantly different immobility scores in the pre-drug FST. In the HI animals, both IMI 5 µg and ALLO 5 µg significantly reduced immobility when compared to the CTR group. IMI-treated rats also showed lower immobility than the ALLO group. In the LI rats, no difference in immobility was found between treatments. In conclusion, two strains of rats with significantly different immobility profiles in the FST were obtained in a relatively short time, after only three generations. Infusion of ALLO and IMI showed a strain-dependent antidepressive-like effect, being detected in the High Immobility animals but not in the Low Immobility animals, which corroborates with the clinical understanding that antidepressants are more useful in severe forms of depression and unable to improve mood in non-depressive subjects.

keywords: depressive disorder; heredity; lateral ventricle; neurosteroid

1. Introduction

Depression is a very prevalent and incapacitating mental disorder that is estimated to affect over 300 million people worldwide, which corresponds to approximately 4.4% of the population [1]. A recent report from the World Health Organization has established depression as the leading cause of disability in the world [2]. Though diverse environmental aspects have been implicated in the causes of depression, its heredity is estimated to be around 40%, indicating the existence of an important genetic factor underlying its etiology [3]. This trait is also observed by studying close family members of depressed individuals, since first degree relatives of probands diagnosed with depression show an increased risk for affective disorders [4]. Its specific genetic mechanisms, however, are yet to be totally discovered. Incomplete efficacy of available antidepressants may be due to variability in the neurobiology of depression [5], which is likely related to its genetic construct. Therefore, new antidepressant agents that act on novel neurotransmitter systems are still needed.

Neurosteroids have been widely implicated in the neurobiology of depression, especially due to their action as modulators of the GABA type A receptor ($GABA_A$-R) [6]. The 5α-reductase inhibitor finasteride, which decreases the concentration of neurosteroids in the brain, has been linked to depressive behavior as a side effect of its application as a treatment for alopecia [7-9] and has been shown to induce depressive-like effects in the forced swim test in mice [10].

Allopregnanolone (3α-5α-tetrahydroprogesterone; 3α-5α-THP; ALLO) is a neurosteroid that is among the most potent positive allosteric modulators of the $GABA_A$-R [11]. It has been reported that allopregnanolone levels are decreased in the cerebrospinal fluid and plasma of depressed individuals [12-14] and that successful treatment with antidepressants reverses its levels back to normal [12, 13]. Animal studies have corroborated this finding, since olfactory bulbectomized rats – a well-validated depression model – have decreased allopregnanolone levels when compared to sham-operated rats [15]. Furthermore, subchronic treatment with antidepressants increases allopregnanolone levels in rats and mice [16, 17]. Intrahippocampal [18] and intra nucleus accumbens infusions of ALLO [19, 20], reduces the immobility time of rats in the forced swim test.

It has also been observed that overexpression of the 18 kDa translocator protein, which is crucial to neurosteroid synthesis, increases allopregnanolone levels and produces an antidepressive-like effect in mice [21]. In addition, when allopregnanolone is infused in the hippocampus [18] or in the nucleus accumbens [19, 20], it reduces the immobility time of rats in the forced swim test. In learned helplessness rats, hippocampal and amygdalar infusion of allopregnanolone also produce antidepressive-like effect [22]. Also, intracerebroventricular infusion of allopregnanolone in mice produces an antidepressive-like effect in the same behavioral test [23]. This study aimed to generate two strains of rats selectively-bred for extreme immobility duration and non-immobility in the forced swim test and to verify the behavioral effects of allopregnanolone in each of those strains, in comparison to imipramine.

2. Materials and methods

2.1 Animals

Wistar adult rats (40 males and 40 females in the first generation) were obtained from the Animal House of Fundação Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). After an initial behavioral selection and breeding of 8 males and 8 females of these F0 animals with higher or lower immobility scores, 60 males and 60 females of the first generation were used and bred to obtain 60 males of the second generation. Offsprings were limited to 10 pups. All animals were housed in groups according to their ages. Groups of three adults were kept in polypropylene cages (25 × 35 × 35 cm) with wood shavings as bedding. When surgery was performed, animals were subsequently maintained in isolated cages. Food and water were available *ad libitum*, and the animals were maintained in a temperature-controlled room (22 ± 2 °C) under a light–dark cycle (lights on from 5 AM to 5 PM). All *in vivo* experiments followed the guidelines of the International Council for Laboratory Animal Science and were approved by the Ethical Committee for the Use of Animals in Research of UFCSPA (process number 15-163). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2 Forced Swim Test

A slightly modified version of the Porsolt Forced Swim Test (FST) was used [24-26], in which the animals were individually introduced in opaque glass tanks (25 x 25 x 40 cm) filled with 28 cm of water at the temperature of 25 ± 1° C. Two swim sessions were conducted: an initial 15-min training section followed 24 h later by a 5-min test section that was video recorded for behavioral analysis. Following both sections, rats were removed from the tanks, carefully dried with towels, and warmed under a heated lamp for 10 min and then returned to their home cages. Behaviors were analyzed using the Behavsoft® software (patent pending) by a trained researcher that was blind to the different treatments. Behavsoft® was developed by our research group and allows viewing and analyzing the video recordings in a single interface using keyboard shortcut commands [27]. In addition to immobility duration, detailed behaviors of mobility (climbing and swimming durations) and number of head shakes were also analyzed, as in Ferigolo and colleagues (1998) [26]. Rats were submitted to non-drug FSTs at PND 65±5.

2.3 Behavioral selection and breeding

All animals of the first generation (F0) were submitted to the FST for screening of the immobility behavior. Rats that scored one standard deviation above or below the mean were selected for the High Immobility (HI; n = 8 males and 8 females) group and Low Immobility (LI; n = 8 males and 8 females) group, respectively. Males and females from the HI or the LI selection were paired for breeding inside their groups and the subsequent generation (F1) was submitted to the FST screening process when adulthood was reached. The high immobility and low immobility selected animals were bred to give origin to the second generation (F2), which was also evaluated in the FST when adults to confirm behavioral selection, as in the previous generations. For mating, a male and a female rat were placed in a cage for two weeks. All procedures to confirm pregnancy, date of birth and weaning were maintained throughout the study. Consanguinity was avoided by matching animals that were least related to one another as possible and sibling mating was not conducted. Male rats of F2 were used in the drug experiment after confirmation of HI or LI behavior.

2.4 Stereotactic surgery

Male rats from F2 were anesthetized with xylazine HCl (15 mg/kg) and ketamine HCl (100 mg/kg) i.p. and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A guide cannula was placed in the right lateral ventricle (anteroposterior: +0.26 mm from bregma; lateral: +1.1 mm from bregma; vertical: −3.5 mm from dura mater according to Paxinos and Watson, 1998 [28]) to diffuse the drugs through the ventricular system. The cannula was fixed to the skull with one screw and dental cement. Surgeries were performed 7±2 days prior to the training day component of the FST.

2.5 Drugs

The vehicle solution consisted in a 20% (w/v) 2-hydroxypropyl-β-cyclodextrin (Fluka-Sigma-Aldrich, St. Louis, MO, USA) solution prepared in artificial cerebrospinal fluid (NaCl 147 mM; CaCl2 2.3 mM; KCl 4 mM; MgCl2 0.9 mM; pH 7.1–7.3), in which both allopregnanolone (Sigma-Aldrich, St. Louis, MO, USA) and imipramine hydrochloride (Aspen Pharma, Serra, ES, Brazil) were dissolved to obtain a concentration of 2.5 μg/μL. The animals of each immobility group were randomly divided into three subgroups. Allopregnanolone (5 μg/rat), imipramine (5 μg/rat) or vehicle were infused in a volume of 2 μL per rat at a constant rate of 0.5 μL/min through a microperfusion pump (EFF 311; Insight Ltda., Ribeirão Preto, SP, Brazil) connected to a 27-gauge needle that was introduced 0.2 mm below the end of the guide cannula. To avoid reflux, the injection needles were removed from the guide cannula 1 min after the end of the infusions. Allopregnanolone, imipramine or vehicle solution were administered three times: 24, 5 and 1 h before the test section of the FST. Each experimental group included 9–11 rats (PND 90±5) for the behavioral tests.

2.6 Cannulae placement verification

Rats that were submitted to intracerebroventricular infusion of drugs were euthanized by decapitation immediately after the FST. Their brains were quickly removed, frozen in liquid nitrogen and stored at -80° C. Posteriorly, brains were briefly thawed and sectioned using a rat brain slicer 1.0 mm (Zivic, Pittsburgh, PA, USA) in the cannula incision area and photographed for macroscopic placement analysis.

2.7 Statistical analysis

All data were compiled with the SigmaPlot™ software (Jandel Scientific Co., v. 11.0, San Jose, USA) for posterior statistical analysis. A one-way ANOVA was used to compare immobility in F0 versus each immobility-selected strain in F1 and F2 combined with a two-way ANOVA to compare immobility scores among these groups (factors: generation versus strain). Males and females were analyzed separately. A one-way ANOVA was used to determine if there was an effect of intracerebroventricular infusion of allopregnanolone and imipramine in HI and LI rat strains. In these cases, Tukey's test was used as a *post hoc* when appropriate. Prior to the ANOVA test, a Normality test (Kolmogorov-Smirnov) and an Equal Variance verifying test were conducted. Data that contained variables that did not assume normal distribution were submitted to a Kruskal-Wallis test followed by Dunn's method. Pearson's correlation test was used to assess the association between immobility duration and head shake counts in screening FSTs. Differences were considered significant when $P < 0.05$. Results of data that assumed normal distribution are shown as mean ± standard error of the mean. Results of data that did not assume normal distribution are shown as median (Mdn) followed by its 1st (Q1) and 3rd (Q3) quartiles.

3. Results

3.1 Breeding of behaviorally selected rats

Immobility mean duration for males in the screening FSTs are shown in Fig. 1. When comparing the immobility of the initial population of F0 to the F1 subgroups, no statistically significant difference was found (F0 *versus* HI: $P = 0.321$; F0 *versus* LI: $P = 0.917$). These HI and LI subgroups of F1 were, however, significantly different from each other ($P = 0.007$). When comparing F0 against F2 subgroups, there was a significant increase in HI immobility scores ($P < 0.001$), while LI scores remained unchanged ($P = 0.999$). Immobility subgroups in F2 were once again significantly different from each other ($P < 0.001$). The change in immobility duration of selectively bred generations was

statistically significant in HI rats, which had higher scores in F2 when compared to F1 ($P < 0.001$). No difference was found when LI rats from F1 and F2 were compared ($P = 0.278$). In females, all comparisons failed to reach statistical significance results not shown).

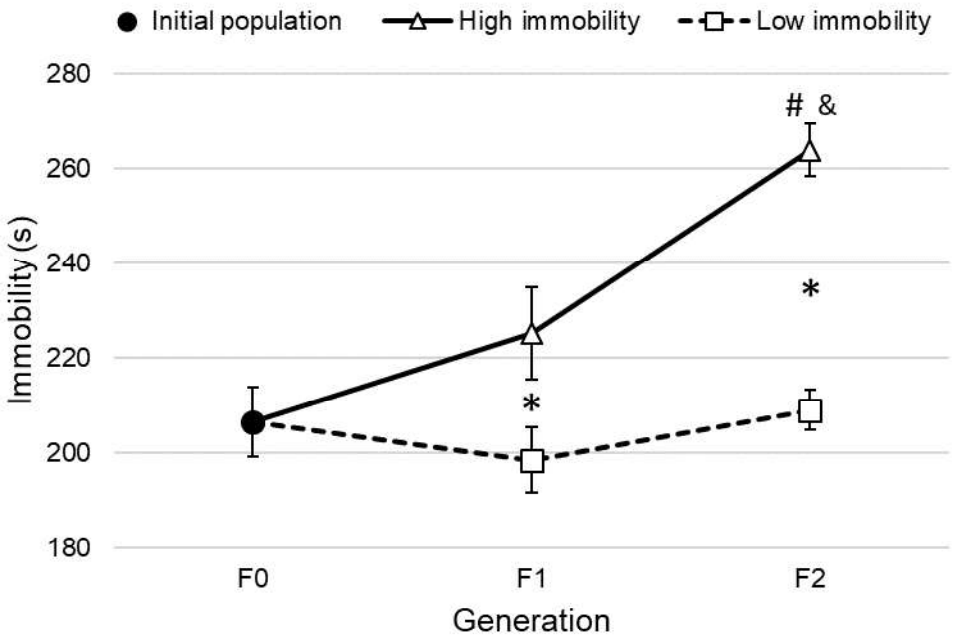

Fig. 1: Immobility scores of male rats throughout three generations. Data presented as mean ± standard error of the mean. One-way ANOVA (F0 *versus* individual groups) and two-way ANOVA (generation x strain) followed by Tukey's test. *$P < 0.01$ when comparing High immobility vs Low Immobility; $^{\#}P < 0.001$ when compared to the initial population (F0); $^{\&}P < 0.001$ when compared to F1 in the same immobility group. Group sizes were n = 40 in F0 and n = 26–32 in the other immobility subgroups.

The detailed behaviors analyzed in the FST did not show normal distribution in any of the variables tested (*P*-value in the Kolmogorov-Smirnov normality test < 0.05) and are represented in Table 1. In F2, we found that climbing behavior was significantly different between immobility groups ($P < 0.05$), as well as swimming ($P < 0.05$). Head shake count was higher in LI rats in F1 ($P < 0.05$) and F2 ($P < 0.05$). There was a moderate negative correlation between head shake counts and immobility duration (r = -0.444; $P < 0.001$).

3.2 ALLO treatment effects in the HI and LI groups

Because a statistically significant difference in immobility scores was found between HI and LI rats in the pre-drug FST in F2 (as shown in section 3.1), these groups were analyzed independently. In the HI rats (Fig. 2A), intracerebroventricular infusion of drugs decreased the immobility time (P < 0.001). Imipramine infusion, as expected, decreased the immobility time by 37% when compared to controls (P < 0.001). Allopregnanolone infusion was also able to reduce immobility time when compared to controls by 17% (P = 0.018). Interestingly, there was a statistically significant difference between allopregnanolone and imipramine treatment groups (P = 0.008), showing a distinct magnitude of effect for each treatment.

The intracerebroventricular infusion of allopregnanolone or imipramine did not produce a statistically significant change in immobility duration in LI rats, as seen in Fig. 2B (P = 0.341).

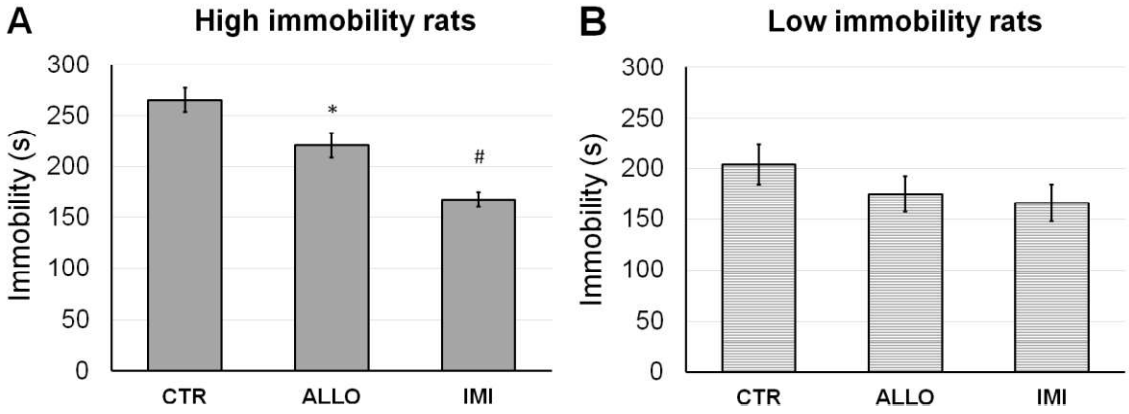

Fig. 2. Effect of intracerebroventricular infusion of allopregnanolone (ALLO), imipramine (IMI) or vehicle (CTR) on immobility time in the forced swim test of high immobility (A) and low immobility (B) F2 male rats. Results shown as mean ± standard error of the mean. One-way ANOVA followed by Tukey's test. *P < 0.05 when compared to CTR; $^{\#}P$ < 0.05 when compared to ALLO and CTR. Each group contains 9–10 rats.

Regarding detailed behaviors of HI rats in the drug FST, only imipramine increased climbing and head shakes when compared to controls (P < 0.05), and both treatments increased swimming time when compared to controls (P < 0.05). In LI rats, no statistically significant difference was found after

allopregnanolone or imipramine infusion in specific behaviors observed in the FST.

Table 1. Detailed behaviors in the forced swim test.

Generation	Immobility group	infusion	Swimming (s) Mdn (Q1 – Q3)	Climbing (s) Mdn (Q1 – Q3)	Head shakes Mdn (Q1 – Q3)
F0	n/a	n/a	26 (8 – 49)	61 (32 – 77)	3 (1 – 7)
F1	High	n/a	12 (0.5 – 29)	51 (28 – 87)	1 (0 – 10)
	Low	n/a	24 (8 – 41)	79 (61.5 – 95.5)	16 (4.5 – 33)$^{\#}$
F2 (pre-drug)	High	n/a	7 (0 – 16)*	23 (7 – 43)*	0 (0 – 6)
	Low	n/a	22 (10 – 45)	58.5 (45 – 77)	21 (7 – 40)$^{\#}$
F2 (post-drug)	High	CTR	4.5 (2 –24)	16.5 (0 – 30)	0.5 (0 – 8)
		ALLO	38.5 (24 – 70)$^{\&}$	22.5 (10 – 55)	2 (1 – 30)
		IMI	76 (40.5 – 89.5)$^{\&}$	49.5 (42.5 – 77)$^{\&}$	23 (11 – 43)$^{\&}$
	Low	CTR	27.5 (8 – 69)	50.5 (0 – 80)	7.5 (1 – 32)
		ALLO	34 (25.5 – 65)	58 (41 – 88)	14 (5 – 35)
		IMI	26 (19 – 100)	67 (28 – 98.5)	19 (4 – 31)

Mdn: median; Q1: first quartile; Q3: third quartile; ALLO: allopregnanolone; IMI: imipramine; CTR: vehicle; *P < 0.05 when compared to LI in the same generation; $^{\#}P$ < 0.05 when compared to F0 in the same immobility group; $^{\&}P$ < 0.05 when compared to CTR in the same immobility group.

4. Discussion

In this work, we were able to generate two behaviorally different rat strains after only three generations of selective breeding for immobility behavior in the FST. Weiss and colleagues (1998) [29] have achieved the same goal by breeding behaviorally selected Sprague-Dawley rats in single-session version of the FST throughout 18 generations. In their experiment, both high and low immobility subgroups differed significantly from the original stock since the first generation bred, indicating that few generations are needed to reproduce the hereditability of depressive-like behaviors. Another study using Wistar-Kyoto

rats selectively bred for immobility time in the FST found results similar to ours, in which F1 rats differed between immobility subgroups. As in our study, only rats selected for high immobility eventually reached statistically significant difference from the initial population [30]. We observed the same pattern, since immobility time of LI rats failed to differ from that of the initial population. These results suggest that the low immobility behavior is a more difficult trait to select by breeding than the high immobility behavior, perhaps because these rats may represent the "normal state" of behavior in the FST. We hypothesize that the genetics underlying the behaviors observed in this kind of studies relate more to the emergence of the behavioral despair state [24] of HI rats rather than being able to provide a motivational aspect to the LI rats, but lack of specific genetic markers for these behaviors make this difficult to confirm.

One behavior that did change in LI rats was the head shake count, which was not only higher than the HI rats in F1 and F2, but also higher than the initial population in F2. Unfortunately, interpretation of this behavior in rats is still limited, but it is believed to be linked to an hyperactivity of the serotoninergic system [31]. In our results, it seems to work as an inverse marker for depressive-like behavior and could be pointing to a higher resilience of LI rats towards a depressive-like state.

One aspect in which our study differed from the ones listed above [29, 30] is that we did not find any changes in the behaviors in the FST in females in the different generations. High variability of behaviors due to the estrous cycle stage is a probable explanation, since it has been observed that the phase of the menstrual cycle influences the expression of immobility of rats in the FST [32] and the risk of depressive behaviors in women [33]. On the other hand, the direct effect of estrous cycle phase in the FST seems to be minor if no drug therapy is being tested [34, 35]. Since neither this study nor the similar studies listed above did control the estrous phase as a factor, this could be faced as a limitation to be corrected in future research.

In the main experiment of our study, we infused either vehicle, allopregnanolone or imipramine to the lateral ventricle of F2 males. Since we successfully obtained two strains of immobility-selected male rats, we analyzed HI and LI separately and made no further statistical comparisons between these subpopulations regarding response to treatments. Concerning the results from

the HI strain, imipramine was used as a positive control group, since this antidepressant has been reported to effectively reduce immobility time in the FST [32]. As expected, IMI infusion reduced the immobility time in the HI strain by nearly 100 seconds (37%). Classic antidepressants have been shown to successfully reduce immobility in other rat strains bred for high immobility [29, 30]. All three lines, including ours, are very effective in detecting the positive effects of antidepressants [36], which may indicate that they model a rather severe state of the depressive disorder. That may be supported by the clinical literature that shows that the effect of antidepressants – including imipramine – is more evident in patients with more severe forms of depression [37]. Regarding detailed behaviors in the FST, imipramine infusion increased climbing and swimming time in HI rats. A similar effect was found when imipramine was administered intraperitoneally at a dose of 10 mg/kg, in which both behaviors were slightly increased but only reached statistical significance in total mobility time [26]. Interestingly, this same work found a significant decrease in the head shake frequency caused by imipramine [26], which was the exact opposite effect we observed in our experiment. Though evidence is scarce, head shake has been shown to increase after stress input and may be reduced by antidepressive treatment with desipramine [38].

Intracerebroventricular infusion of allopregnanolone was also able to reduce immobility time in a more modest, yet statistically significant magnitude of 17% in HI rats. This effect is smaller than what was found by Nin and colleagues (2012) [20] when the same dose of allopregnanolone was infused into the nucleus accumbens. It is probable that the diffusion of the drugs in the ventricular system led to dilution, and therefore action of smaller doses in several brain regions, leading to a milder effect of allopregnanolone. However, we cannot discard the possibility that the HI rats are more resistant to the antidepressant effect of allopregnanolone, contrasting with the effects of other antidepressants in this model. We also observed a lack of effect on climbing behavior combined with an increase in swimming time caused by allopregnanolone, which is also contrasting to results found after allopregnanolone infusion in the nucleus accumbens, in which climbing but not swimming behavior was increased [19, 20]. Climbing behavior in the FST has been used as a marker for norepinephrine alterations caused by

antidepressants [25]. However, in different occasions it has been seen that GABA A receptor interacting drugs, including allopregnanolone[20] and clonazepam [39], also increase climbing and not swimming in the FST, and it remains to be defined if any noradrenergic or GABAergic mechanisms are involved in the antidepressant-like effects of allopregnanolone.

In the LI rats, none of the drugs tested produced an antidepressive-like effect or changes in the detailed behaviors in the FST. This probably corroborates with the concept that antidepressants will not improve mood in non-depressed individuals. An interesting observation is that LI rats were behaviorally very similar from the non-selected initial population, in which these drugs would be expected to reduce immobility time in the behavioral test used. This may indicate that genetic and/or epigenetic changes are present in these animals, but do not result in behavioral changes in screening FST rather than granting resilience to changes made by antidepressive agents. Will and colleagues (2003) also found very modest or no effect of antidepressants administered to the strain selected for low immobility, even though this strain also did not differ from the original stock [30]. And when a strain bred for low immobility in the FST does differ from the original stock, nearly no drug-induced antidepressant effect was observed in these animals [40], providing more evidence to the hypothesis raised above.

5. Conclusion

This study provides evidence for a hereditary animal model of depression, generating two rat strains that are behaviorally distinct in the FST and that respond differently to the treatments with classical and novel antidepressant drug therapies. Our findings show a successful antidepressant-like effect of allopregnanolone on rats selectively bred for higher depressive-like behavior in the FST, indicating that the role of neurosteroids such as allopregnanolone, is relevant for treatment and almost comparable to imipramine especially in the most severe presentations of depression. Further studies should be conducted to assess the neurochemistry of these two strains of rats and how they are modulated by the treatment with allopregnanolone compared to imipramine.

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6. CONCLUSÕES

Neste trabalho, foram produzidas duas linhagens de ratos que, através do cruzamento seletivo de características comportamentais tipo-depressivas por três gerações, deram origem a duas linhagens comportamentalmente diferentes entre si e da população inicial. Esta última comparação foi verdadeira, porém, apenas no caso dos ratos selecionados para alta imobilidade. Além disso, observamos um efeito diferenciado das drogas administradas via intracerebroventricular em cada linhagem: tanto o antidepressivo tricíclico imipramina – utilizado como controle positivo – quanto a alopregnanolona produziram um efeito tipo-antidepressivo nos animais de alta imobilidade, mas não nos ratos selecionados para baixa imobilidade. Estes resultados indicam que há uma modulação genética da resposta a antidepressivos, já que os ratos de baixa imobilidade apresentaram uma resistência mesmo à ação de antidepressivos que classicamente mostram efeito no teste utilizado. Nos ratos de alta imobilidade, o efeito tipo-antidepressivo da alopregnanolona demonstra que a modulação do sistema GABAérgico é importante na fisiopatologia do comportamento tipo-depressivo destes ratos, já que é a primeira vez que o efeito tipo-antidepressivo deste neuroesteroide é testado neste modelo animal. Resta saber como estas drogas alteram fatores neuroquímicos e epigenéticos relacionados à plasticidade de receptores e proteínas neurotróficas subjacentes ao efeito tipo-antidepressivo que provocam neste modelo.

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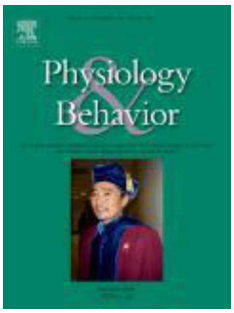

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[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000. Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304. Reference to a website:
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ANEXO II

Carta de aprovação do projeto de pesquisa no CEUA-UFCSPA (15-161)

UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
UFCSPA

A Comissão de Ética no uso de Animais, analisou o Projeto:

Projeto: 15-161 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
HELENA MARIA TANNHAUSER BARROS
MAURÍCIO SCHÜLER NIN
FELIPE BORGES ALMEIDA

Título: EFEITOS DA ALOPREGNANOLONA SOBRE O COMPORMENTO TIPO DEPRESSIVO E SISTEMA GABAÉRGICO DE RATOS COM PERFIS DISTINTOS DE IMOBLIDADE DO NADO FORÇADO.

Este projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim com eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 06 de novembro de 2015.

Katya V. Rigatto
Coordenadora do CEUA
UFCSPA